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Mechanism and Regulation of Eukaryotic Protein Synthesis

WILLIAM C. MERRICK

Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

INTRODUCTION

This review has two goals. The first is to provide a rather simple base to allow those less familiar with the intricate details of protein synthesis to appreciate the physical processes going on. This should lend itself to a chance at understanding the very complex process of regulating translation. The second goal is to provide some insight into the current problems, holes in my arguments, points of interest for further study, etc., that more fully explore the state-of-the-art data currently available. However, this will still be intended for the nonexpert, and I hope the experts will not be too upset that I have chosen to present a biased (but maybe educated) view and that not all of the world's literature has been cited. Nonetheless, apologies for my ignorance or omissions in advance. To atone for some of my sins, numerous other review articles are now cited (16, 69, 71, 110, 131, 145, 195, 207, 213, 237, 247, 253, 278).

EUKARYOTIC PROTEIN SYNTHESIS: THE SIMPLE VIEW

Trying to present the simple view is a bit like describing football or baseball to someone who doesn't know the game. In this regard, Fig. 1 and 2 are examples of the players in action and Table 1 is the handy reference score card. Perhaps the simplest approach is to describe each factor in isolation and then describe their use in the process of initiation or elongation (termination will receive rather little comment, and interested readers are suggested to monitor reports from the Caskey and the Tate laboratories for the latest word). It should be noted that the "old" nomenclature (7) will be used because this most directly relates to publications in the field.

eIF-1

Eukaryotic initiation factor type 1 (eIF-1) is one of the smallest and least well studied initiation factors which has been purified by several laboratories (15, 260, 300). By gel filtration and sodium dodecyl sulfate (SDS)-gel electrophoresis, eIF-1 appears to be a single polypeptide of molecular weight 15,000. The reason that this protein has not been well studied reflects in part the slight stimulation that it gives to protein synthesis, often 20% or less (15), and the observation that it provides this slight stimulation to several steps rather than just one. This is in fact the characteristic that one would describe of a ribosomal protein, although, by definition, the

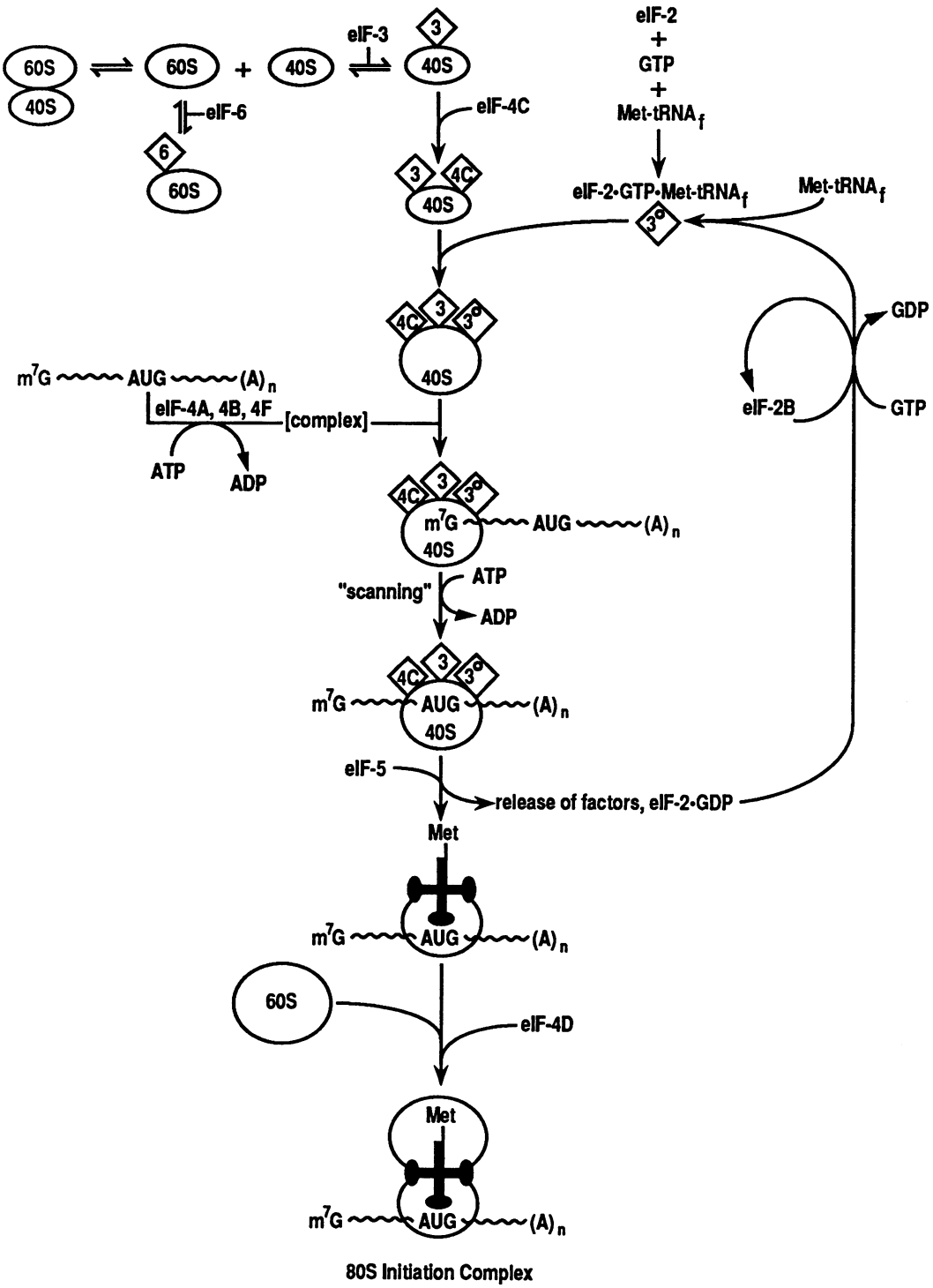


FIG. 1. Flow scheme for formation of 80S initiation complexes. This flow scheme is discussed in the text and is to serve only as a convenience for placing individual factors and assessing their role in 80S complex formation. Reality is likely to be more complicated.

initiation factors cycle on and off the ribosome and are not permanent residents. In this regard, radiolabeled eIF-1 did not appear to form a stable complex with 40S, 60S, or 80S ribosomes and thus, like all the translation factors to be covered in this review, would appear to cycle on and off the ribosome.

eIF-2

eIF-2 has been purified independently in over 25 different laboratories. In all but a few instances, each laboratory has found eIF-2 to contain three polypeptides with molecular masses of 52 (γ), 38 (β), and 35 (α) kDa. The rare exceptions

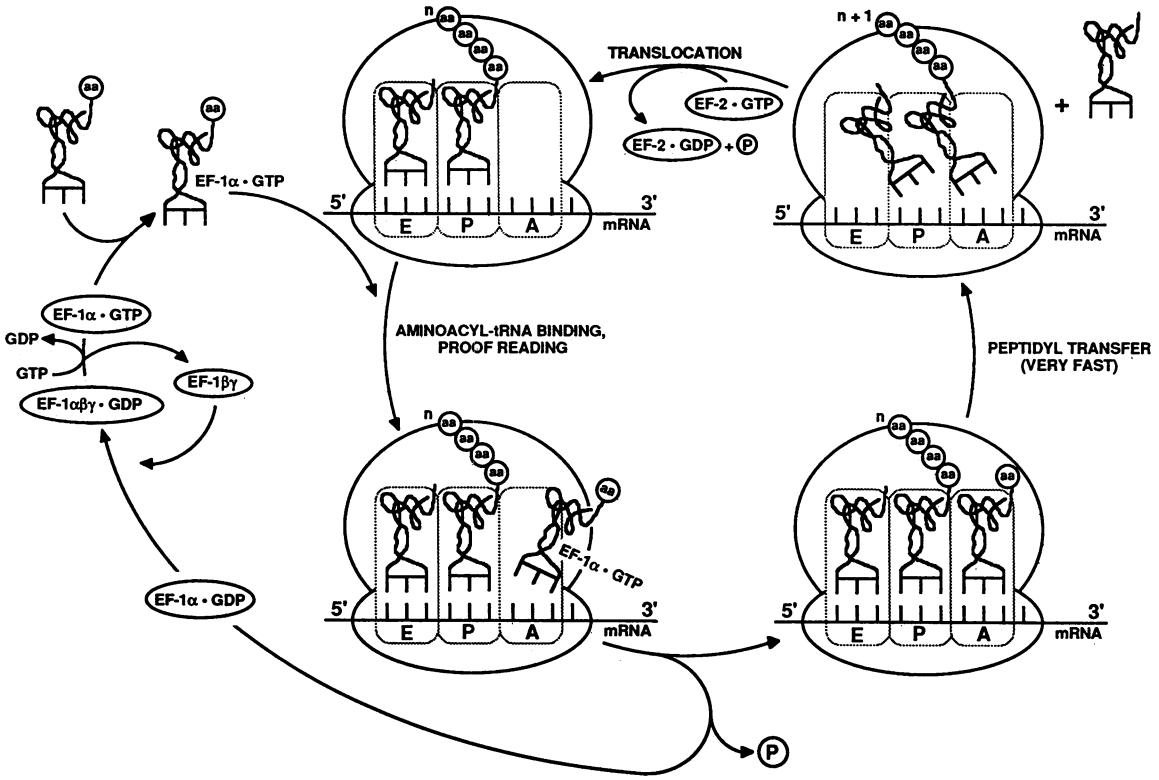


FIG. 2. Flow scheme for the elongation cycle in eukaryotes. This flow scheme is heavily patterned after the "half-site" model of Moazed and Noller (188) and is based on the similarity in structure and function of eukaryotic and prokaryotic elongation factors. The E site is the exit site, the P site is the peptidyl-tRNA site, and the A site is the site for the binding of incoming aminoacyl-tRNAs. The slow steps in the cycle appear to be the binding of the aminoacyl-tRNA and translocation, whereas peptidyl transfer appears to be quite rapid. See the text for details. aa, amino acid.

when a two-subunit preparation has been identified can generally be considered to represent proteolysis of the β subunit, which is very susceptible and, once clipped, readily lost during purification. Alternatively there may have been incomplete resolution of the β and γ subunits by SDS-gels. The use of cDNA cloning has resulted in the isolation and sequencing of the α subunit from yeast cells (*Saccharomyces cerevisiae*) (39) and from rat and human cells (75). Similarly, the β subunit has been cloned from yeast (65) and human (224) cells. Although several groups have obtained partial sequences of the γ subunit, there has been no report of a complete γ sequence. However, recent evidence suggests that the yeast *GCD11* gene may in fact be the γ subunit of eIF-2 on the basis of a 50% or better identity to several long polypeptide sequences from rabbit reticulocyte eIF-2γ (102a). The *GCD11* gene sequence has the distinctive GTP consensus element sequences and appears to be related to EF-Tu, the bacterial protein which functions in the elongation cycle and binds aminoacyl-tRNAs in a GTP-dependent fashion.

The primary function of eIF-2 is to bind the initiator tRNA, Met-tRNA$_i$, in a GTP-dependent manner. As will become apparent below, this is important both for providing an aminoacyl-tRNA in the P site of the ribosome and for identifying the initiating AUG codon. Chemical studies have attempted to identify the subunit in eIF-2 responsible for GTP binding (8, 18, 60, 182). The major site of labeling has consistently been the β subunit, with one exception, when the γ-(*p*-azido)anilide of GTP was used. This reagent specifically labeled the γ subunit of eIF-2 (18), whereas other reagents with reactive groups off the ribose or base portions of either GDP or GTP gave a predominant labeling of the β subunit (8, 18, 60, 182). However, as noted above, the apparent eIF-2γ sequence would suggest that this subunit should be capable of binding GTP. Therefore it is possible that the GTP site is shared between the β and γ subunits (18) or that there are two nucleotide-binding sites on eIF-2.

In a like manner, efforts have been made to identify the subunit(s) of eIF-2 which binds the initiator tRNA. Early studies indicated that cross-linking of the tRNA was predominantly to the β subunit of eIF-2 in the ternary complex (GTP · Met-tRNA$_i$ · eIF-2) (208) or as the ternary complex bound to 40S subunits (307). More recent data obtained by using chemical cross-linking agents have identified peptides in the β (*trans*-Pt) and γ (diepoxybutane) subunits of eIF-2 as cross-linking to the initiator tRNA (136). Given the EF-Tu-like sequence of the γ subunit and the zinc finger motif of the β subunit, it is possible that more than a single subunit is involved in the binding of the initiator tRNA. It might be noted that despite the zinc finger motif, active eIF-2 does not contain zinc (136a). There is no evidence that the α subunit is involved in the binding of Met-tRNA$_i$.

The above data would suggest that the GTP-binding site and the Met-tRNA$_i$-binding site are likely to be shared between the β and γ subunits. There are, however, a series of experiments that report on the isolation of a functional eIF-2 lacking the β subunit (8, 46, 187), although often the specific activity was slightly reduced. This reduction in

TABLE 1. Properties of eukaryotic translation factors[a]

Old nomenclature	New nomenclature	Mol mass (kDa)		Characteristic activity	Covalent modifications[b]	Sequence known	Other comments
		SDS	Native				
eIF-1	eIF-1	15	15	Pleiotropic		No	
eIF-2	eIF-2	36, 38, 55	125	GTP-dependent Met-tRNA$_i$ binding	36 and 38 PO_4	36, 38, 55	Major site of regulation
eIF-2A	eIF-2A	65	65	AUG-dependent Met-tRNA$_i$ binding to 40S subunits		No	Only shown functional in AUG-dependent assays
eIF-2B, GEF	eIF-2B	26, 39, 58, 67, 82	270	Guanine nucleotide exchange factor for eIF-2	67 and 82 PO_4	No	
eIF-3	eIF-3	35, 36, 40, 44, 47, 66, 115, 170	650	Subunit dissociation	35, 44, 47, 66, and 115 PO_4	No	
eIF-4A	eIF-4A	45	45	RNA-dependent ATPase		Yes	Isozymes
eIF-4B	eIF-4B	80	140	Stimulates activities of eIF-4A and eIF-4F	PO_4	Yes	
eIF-4C	eIF-1A	16	16	Stimulates subunit joining		Yes	Alleles/isozymes
eIF-4D	eIF-5A	17	17	Stimulates first peptide bond synthesis	Hypusine	Yes	Isozymes
eIF-4F	eIF-4	24, 45, 220	?	Recognizes m^{7}G cap of mRNA	24 and 220 PO_4	24, 45	Major site of regulation
iso-eIF-4F	iso-eIF-4	28, 80	?	Recognizes m^{7}G cap of mRNA		No	Observed only in plants
eIF-5	eIF-5	150	125	Subunit joining	PO_4	No	
eIF-6	eIF-3A	25	25	Subunit dissociation		No	
EF-1α	eEF-1α	51	51	GTP-dependent binding of aminoacyl-tRNA	CH_3, GPE	Yes	Isozymes
EF-1$\beta\gamma$	eEF-1$\beta\gamma$	30, 48	$(30 + 48)_x$	Guanine nucleotide exchange factor for EF-1α	30 and 48 PO_4	30, 48	
EF-2	eEF-2	95	95	Translocation, ribosome-dependent GTPase	Diphthamide, ADP-ribose, PO_4	Yes	
EF-3	eEF-3	125	125	Ribosome-dependent ATPase and GTPase		No	Present only in yeasts
RF	eRF	54	108	Codon-dependent release of fMet-tRNA from ribosomes		Yes	4 nucleotide recognition

[a] The majority of the characteristics for the translation factors are derived from the references cited in the text for each factor.
[b] PO_4 represents phosphorylation, CH_3 represents methylation, and GPE represents glycerylphosphorylethanolamine.
[c] Isozymes is meant to indicate the presence of two functional genes for that protein which may or may not have the same coding sequence. In addition, because the gene counting has only recently begun, it is possible that factors expressed from more than one functional gene in one organism have only a single functional gene in another (iso-eIF-4F, perhaps). Correspondingly, what is a single gene in one species could have multiple functional genes in another. While the term "isozymes" indicates multiple genes, the lack of that designation does not necessarily mean that only a single functional gene exists for that protein.

specific activity could reflect either the additional handling necessary to get rid of the β subunit or a direct effect of the β subunit. No one has reported on the isolation of an eIF-2$\alpha\beta$ complex, and so it has not been possible to assess the specific loss of functions that might be associated with the γ subunit.

eIF-2B (GEF)

As a product of initiation, a complex of eIF-2 and GDP is released. Inasmuch as eIF-2 has a 100-fold preference for GDP over the substrate GTP ($K_d^{GDP} \approx 10^{-8}$ M) and the off-rate for GDP is slow, protein synthesis would usually be limited by this slow exchange to re-form eIF-2 · GTP and thus allow reutilization of this factor. A similar situation exists in the bacterial elongation cycle, in which EF-Tu also has a 100-fold preference for GDP. To get around this apparent kinetic block to protein synthesis, there are proteins which interact with these factors to facilitate nucleotide exchange. For eIF-2 that protein is guanine nucleotide exchange factor (GEF) or eIF-2B, and for bacterial EF-Tu it is EF-Ts. GEF has been characterized by a number of different laboratories (10, 62, 139, 176, 214, 216, 255, 270). It contains five polypeptides with molecular masses of 82, 67, 58, 39, and 26 kDa and usually is isolated as a complex with eIF-2. One report has also noted the association of NADPH with GEF, which is likely to be involved with the regulation of GEF activity (61). By use of affinity labeling, a GTP-binding site has been identified on the 39-kDa subunit and ATP-binding sites have been identified on both the 58- and 67-kDa subunits (60). The GTP-binding site may be related to the nucleotide exchange reaction. The role of ATP- and NADPH-binding sites is less clear in relation to function, but may be more easily explained in terms of regulation.

eIF-3

eIF-3 is the largest of the initiation factors, with an aggregate molecular mass of about 600 to 650 kDa (15, 111, 254, 260, 265, 300). The mammalian protein contains 8 different polypeptides, whereas the wheat germ protein contains at least 10 different peptides (111). These two proteins are compared in Table 2. As can be seen in Table 2, there is a general agreement in the character of the eIF-3 subunits, with the two exceptions that there appear to be no basic polypeptides (pI > 8) in mammalian eIF-3 and that the

TABLE 2. Subunits of eIF-3[a]

Mammalian eIF-3		Wheat germ eIF-3	
Mol mass (kDa)	pI	Mol mass (kDa)	pI
35	5.0	28	5.5
36	5.9	34	5.4
40	6.5	36	7.1
44	6.3	41	7.2
47	5.7	45	6.1
		56	6.1
66	6–7	83	5.9
		87	5.9
115	5.3	107	>8
170	6.7	116	>8

[a] Values for apparent molecular mass and pI were from references 108a, 111, and 253. The matching of the subunits was based on relative molecular mass and pI.

largest subunit of wheat germ eIF-3 is only about two-thirds the size of its mammalian counterpart. Hydrodynamic analysis of rat liver eIF-3 combined with electron-microscopic results suggest that eIF-3 has the shape of a flat triangular prism with sides of 17, 17, and 14 nm and is 7 nm thick (171).

eIF-4A

eIF-4A is functional as a single polypeptide chain of about 45 kDa (15, 17, 46, 96, 158, 260, 264, 265, 300). This was the first initiation factor for which two separate, functional genes were identified; they are 98% identical if one allows for the most conservative amino acid substitutions (204). Subsequently two identical eIF-4A gene products were identified in yeast cells (167). eIF-4A has been characterized as a single-stranded RNA-dependent ATPase, an activity characteristic of RNA helicases or unwinding proteins (2, 95, 234). Soon other proteins which were homologous to the amino acid sequence deduced for eIF-4A were identified, and a particular sequence motif within this homology was used to identify this group of proteins as the D-E-A-D box family (166). Although an ever-increasing number of these D-E-A-D box proteins are being identified, the largest number so far are associated with mRNA processing (303). On the basis of the biochemical characterization of eIF-4A and a few other D-E-A-D box proteins as well, the entire family have been classified as putative RNA helicases.

A second feature noticed with eIF-4A is that the amino acid sequence appears to be so highly conserved that the sequence for rabbit eIF-4A is 100% identical to that for mouse eIF-4AI (42), and it was subsequently observed for most of the translation factors characterized until then that the amino acid sequences were very highly conserved (183). This general similarity in amino acid sequence, combined with numerous biochemical characterizations, is a cornerstone in the assumption that there is a general mechanism for protein synthesis which applies to all eukaryotes although some differences could arise (e.g., see EF-3 below!).

A third feature which has been noted for the mouse eIF-4A gene products is that the ratio of the eIF-4AI to eIF-4AII mRNAs (and presumably proteins as well) varies about 30-fold depending on the tissue source (204). Within most tissues the apparent eIF-4AI content was similar while the amount of eIF-4AII varied 20-fold and in general could account for most of the aggregate difference in the amounts of eIF-4A in different tissues. The rationale for this difference in eIF-4AI and eIF-4AII levels is not clear. However, the observation that eIF-4F (which contains eIF-4A as a subunit [see below]) appears to prefer (bind more tightly to) eIF-4AII (42) might suggest a possible tissue-specific regulation of protein synthesis. This should also serve as a cautionary note that although the same translation factors may be present in all cells, their relative abundances may not be fixed and thus the translational characteristics of each cell could be qualitatively similar but quantitatively different.

eIF-4B

eIF-4B has been generally characterized from mammalian sources as a dimer of identical subunits of about 80 kDa (15, 180, 260, 300). The same protein has been purified from wheat germ, and the apparent molecular mass is considerably smaller, about 59 kDa (25, 28, 266). A part of this difference in size appears to be an electrophoresis artifact, because the molecular mass of eIF-4B from the cDNA clone for the mammalian protein is 69 kDa (185). Within the coding region, two sequences (AFLGNL and KGFGYAEF) which indicate an RNA recognition motif were identified. It should be noted, however, that most assays of eIF-4B have required other initiation factors to bind mRNA or effect the synthesis of polypeptide chains, and thus a specific function of eIF-4B independent of other translation factors is lacking. The facts that eIF-4B is necessary to observe recycling of eIF-4F (233) and cross-linking of eIF-4A to mRNA (2) and that it associates quite strongly with eIF-4F (97) argue either that eIF-4B has a role in coordinating the activities of other initiation factors or that, in vivo, eIF-4B may cycle in constant association with eIF-4F. However, biochemically the most dramatic effect of eIF-4B is the stimulation of the RNA-dependent ATPase and helicase activities of eIF-4A (3, 156, 245).

eIF-4C

The low-molecular-mass initiation factor (~17 kDa) eIF-4C has been characterized in both mammalian (15, 134, 260, 300) and plant (58, 263, 265) systems. Although this protein can be isolated in an active form as a monomer, it has also been isolated as a high-molecular-mass complex with eIF-5 (260). In the plant system, eIF-4C appears to be the only heat-stable initiation factor, maintaining more than 85% of its activity after being heated to 90°C for 5 min (263). Recent studies involving amino acid sequencing and cDNA cloning indicate that eIF-4C is about 153 amino acids long and that the wheat germ and mammalian proteins are 66% identical and 75% similar when conservative amino acid substitutions are allowed (110a). Although no particular sequence motifs were noted, the protein is curiously polar; 10 of the first 22 amino-terminal residues are basic, and 13 of the 20 carboxy-terminal amino acids are acidic. It was also noted during the protein sequencing that at several positions, two amino acids were found (glutamic and aspartic acids; isolucine and valine), suggesting the existence of multiple functional genes or alleles.

eIF-4D

Like eIF-4C, eIF-4D is a low-molecular-mass (~16-kDa) protein which has not had a specific function assigned but appears to function late in the initiation complex pathway (15, 134). However, one report noted that a major effect of eIF-4D was to shift the optimal Mg^{2+} concentrations for complete polypeptide synthesis although this effect was

minimized by the presence of 0.03 mM spermine (260). This is a rather curious observation, given that a few years later eIF-4D would be characterized as the one protein in the cell which undergoes a posttranslational modification with spermidine to yield the amino acid hypusine [N^{ϵ}-(4-amino-2-hydroxybutyl)lysine] and is apparently the only protein in the cell to undergo this modification (43). This unique labeling with spermidine allowed for the analysis of different eukaryotic organisms, with the result that this protein with its unique modification is conserved among eukaryotes but is not present in eubacteria or archaebacteria (90). The hypusine modification appears necessary for eIF-4D activity (222). The lysine residue in eIF-4D, which is modified, was identified in the sequence T-G-hypusine-H-G-H-A-K (221) and was determined to be lysine 50 when the protein was sequenced and cDNA clones were obtained (277).

Studies on the enzymology of hypusine biosynthesis indicate that it occurs by a two-step process (200, 219, 220) which involves first the transfer of the 4-aminobutyl group from spermidine to the ε-amino group of lysine 50 and then the hydroxylation of the deoxyhypusine to yield hypusine. Current evidence suggests that the formation of hypusine is not regulated but is subject to the availability of spermidine within the cell (218). However, once the modification is accomplished, it does not seem to be reversed (91). Thus, regulation of eIF-4D activity by removal of the modification necessary for activity does not appear to occur.

eIF-4F

Unlike the other protein synthesis factors, which were isolated and defined as a requirement for complete polypeptide chain synthesis, the discovery of eIF-4F was driven by observations in poliovirus-infected cells, in which uncapped poliovirus mRNAs were translated in preference to the nondegraded, capped host mRNAs (72, 81). Shortly thereafter an assay was developed which allowed for the measurement of interaction of the 5′ m^7G group with protein components by use of oxidized mRNAs (280). An assay specific for eIF-4F resulted from the observation that the addition of crude initiation factors could restore normal host mRNA translation (106). This allowed for the final purification and characterization of eIF-4F, a protein composed of three subunits of molecular masses 220, 45, and 24 kDa (97). Subsequent studies indicated that the inactivation of eIF-4F in poliovirus (77), rhinovirus (76), and foot-and-mouth disease virus (54) is the result of a proteolytic cleavage of the 220-kDa subunit. While the proteolyzed p220 inactivates eIF-4F for the translation of host mRNAs, the proteolyzed form does appear to specifically stimulate the translation of poliovirus mRNA (30).

The function of eIF-4F in mRNA utilization is discussed below; however, it is appropriate to give some description of the individual subunits at this point. The small subunit, occasionally referred to as eIF-4E, has been cloned from human (248), yeast (5), and mouse (4) cells with extensive sequencing of peptides from the rabbit protein (248). This subunit appears to be uniquely responsible for recognition of the m^7G cap structure at the 5′ end of eukaryotic mRNAs (71, 95, 237, 279, 282, 304). On theoretical grounds, it was proposed that the recognition of the m^7G cap might occur through π-π stacking interactions on the basis of observations of m^7G and tryptophan in solution and as cocrystals (118), and results of biophysical studies have been consistent with this suggestion (31, 177). A very recent report makes it likely that the key residues involved are Trp-102 and Glu-105 in the human protein (290), although it should be noted that changes at a number of residues in eIF-4E lead to a loss of biologic activity (4, 6).

As mentioned above, the 45-kDa subunit of eIF-4F is eIF-4A (42), although it is possible that the relative ratio of eIF-4AI and eIF-4AII varies depending on the source of the material chosen for the isolation of eIF-4F. It should be noted that several investigators have isolated a form of eIF-4F which lacks this subunit (27, 30, 78, 234). The isolated protein appears completely active in translation, although it should be noted that eIF-4A would be present in the translation reaction. In particular, it has been shown that chromatography on phosphocellulose will cause the release of the eIF-4A subunit (234). This characteristic is similar to the observed loss of the σ subunit of bacterial RNA polymerase and may indicate that eIF-4A normally cycles in and out of complexes with the 220- and 24-kDa peptides.

As a final note, two forms of eIF-4F (both lacking the 46-kDa peptide) have been purified from wheat germ cells and have been shown to be antigenically unrelated (27). The "true" eIF-4F molecule contains two subunits of 220 and 26 kDa, and the iso-eIF-4F molecule contains two subunits of 80 and 28 kDa. Both of the small subunits interact with the m^7G cap and in general are equally functional in a reconstituted wheat germ assay system. At present it is not clear whether other systems also contain isozymes for eIF-4F function, although evidence for isozymes or alleles of other factors (eIF-4A, eIF-4C, eIF-4D, and EF-1α) have been reported; however, in these latter cases the isozymes (allelic variants) were highly similar in primary sequence. Given the original difficulty in the purification of eIF-4F, it is entirely possible that an iso-eIF-4F molecule could be present in mammalian systems as well.

eIF-5

Relatively little work has been done on eIF-5 since its initial purification (15, 29, 184, 260), at which time the protein was characterized as a single polypeptide chain of 125 kDa with a ribosome-dependent GTPase activity, appropriate for its proposed role in subunit joining. The one observation made was that this was catalytically the most active of the translation factors and that usually 100 ng or less would saturate most biochemical assays. This high level of activity was unfortunately mirrored by a relatively low abundance, making it a difficult protein to purify. However, more recent work has cast doubts on the true molecular nature of eIF-5, suggesting that it is more likely to be a 60-kDa protein (85). There is currently no resolution of these differences. Whether one preparation was contaminated, the other was proteolyzed, or isozymes of different molecular forms exist (as with wheat germ eIF-4F and iso-eIF-4F) awaits further study. However, it should be noted that to date, there has been no difference in the biologic properties ascribed to the two different forms of eIF-5.

eIF-6

As was true for eIF-5, there has been relatively little new work on eIF-6 since its original purification and characterization from wheat germ (246), calf liver (294), or rabbit reticulocytes (235). This protein appears functional as a single polypeptide of 25 kDa. As noted below in the section directly addressing mechanism, eIF-6 appears to provide most of the normal ribosomal "antiassociation" activity, although eIF-3 appears to play a role as well.

EF-1α

Elongation factor type 1α (EF-1α) is perhaps the most widely studied translation factor in eukaryotic systems. This is most probably a reflection of the advanced analysis of the three-dimensional structure of its prokaryotic counterpart (EF-Tu) (126, 137, 151), the extreme interest in GTP-binding proteins as relates to signal transduction and oncogenesis (21, 40, 59, 126, 151, 212, 293), and the fact that EF-1α is one of the most abundant cytoplasmic proteins, constituting between 3 and 10% of the soluble protein. At the last check of the EMBL data base, over 25 EF-1α sequences had been reported, representing more than 15 different species. Beyond this, three different EF-1α proteins have been chemically sequenced (34b, 55, 183, 295), and in this process, different posttranslational modifications were found; they are illustrated in Table 3. Of particular interest is the methylation at position 55, which is close to, but not exactly matching, the Lys-56 residue in *Escherichia coli* EF-Tu, which is more active than its undermethylated counterpart (285). When the three species are compared, only the trimethyllysine at residue 79 is conserved although the most carboxy-terminal methylated lysine is reasonably conserved. In contrast to this simple modification, a more complex modification was noted in mouse and rabbit EF-1α, the addition of glycerylphosphorylethanolamine (55, 309). This modification is not present in yeast EF-1α (34b) but may be present at position 374 (but not position 301) in *Artemia salina* EF-1α (6a). The role of this latter modification is especially unclear given that yeast EF-1α is just as active as rabbit EF-1α when compared in a standard rabbit reticulocyte elongation assay.

There are two additional points of interest. EF-1α genes appear to be present in more than one copy and, in some instances, undergo cell type or stage-specific expression in *Saccharomyces cerevisiae* (201, 258), *Mucor racemosus* (169), *A. salina* (164), *Drosophilia melanogaster* (115), and *Xenopus laevis* (64, 147). The number of functional genes in humans is unknown owing to the complication of sorting out authentic genes from pseudogenes, the estimate for which is about 40 copies in the human diploid complement (291). This large number of pseudogenes in part reflects the rather high percentage of EF-1α mRNA present in cells to encode this abundant protein. In the same context, it was noted that by the use of nuclear extracts from HeLa cells, the human EF-1α promoter appears to be the strongest yet described (291). For those dreaming of growing old gracefully, the overexpression of EF-1α in fruit flies has been shown to enhance the life span, especially at elevated temperature (268). This may reflect the observation made with yeast cells that increased levels of EF-1α yielded increased translational fidelity in vivo (281).

The second area of special interest in EF-1α is that it appears to have a number of other possible functions. These include being part of messenger ribonucleoprotein particle (mRNP) complexes (94), being part of the valyl-tRNA synthetase complex (13, 192), binding to actin (310), being associated with the endoplasmic reticulum (105) or the mitotic apparatus (210), and being involved in protein degradation (261a) or ribosome association (108). Although one has a certain skepticism about the specificity of some of these possible interactions, given the abundance of EF-1α and its pI of about 9.5, it is clear that these authors are aware of these difficulties and have taken precautions to avoid artifacts. The real test, however, will be to show some biological relevance for these observations.

TABLE 3. Posttranslational modifications to EF-1α

Cell type	Sequence with following modification (modified residue no.)[a]:									
	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	GPE	CH_3	GPE	CH_3
Rabbit	LIYKCGG (30)	◆◆◆ GIDKRTI (36)	◆◆ GSFKYAW (55)	◆◆◆ SLWKFET (79)	◆◆ YSDKRYE (165)	VTRKDGN (219)	▼ ALSEALP (301)	◆◆◆ VSVKDVR (318)	▼ KFAELKE (374)	DGPKFLK (392)
Brine shrimp	LIYKCGS (30)	◆◆◆ SIDKRTI (36)	◆ GSFKYAW (55)	◆◆◆ ALWKFET (79)	FSEARFE (165)	◆◆◆ IERKEGK (219)	ESLEQAS (300)	◆◆◆ FNVKNVS (313)	E KFAQIKE (374)	AEPKFIK (392)
Yeast	◆ LIYKCGG (30)	GIDKRTI (36)	GSFKYAW (55)	◆◆◆ ALWKFET (79)	WDESRFQ (163)	KETKAGV (217)	QLEQGVP (299)	◆◆ VSVKEIR (316)	RFDELLE (372)	◆ DHPKFLK (390)

[a] CH_3 indicates the presence of a possible methyl group on the ε-*N* of lysine, and ◆ indicate the number of methyl groups observed. GPE indicates the presence of a possible glycerylphosphorylethanolamine residue, and ▼ indicates residues where it has been observed. The small E above glutamine 374 in the brine shrimp sequence indicates that glutamic acid was inferred from the cDNA sequence, but that glutamine was found by chemical sequencing of the protein. This residue may be modified as observed in the mammalian protein.

EF-1βγ

EF-1βγ, a complex of two polypeptide chains, serves the same function as its prokaryotic homolog, EF-Ts, to facilitate nucleotide exchange (207, 238, 247), although it has been shown that the γ subunit (generally about 35 kDa) is sufficient for this activity (119, 275). Besides the β subunit (about 48 kDa), several different laboratories have reported an additional subunit (δ, about 32 kDa). Perhaps the most unusual aspect is that EF-1βγ (or EF-1αβγ) can exist in very high molecular mass aggregates with molecular masses at or exceeding 2,000 kDa (33, 44, 88, 153, 162, 199). Despite the very large number of sequences known for EF-1α, the amino acid sequence for EF-1β is known only from brine shrimp (174). However, sequences for brine shrimp (173), *X. laevis* (124), human (256), and pig (256) EF-1γ have been determined. Despite the relatively high conservation of amino acid sequence homology between EF-1α and EF-Tu, neither EF-1β nor EF-1γ shows homology to bacterial EF-Ts.

EF-2

EF-2 is a single polypeptide chain with a molecular mass of 95 kDa. It is responsible for the GTP-dependent translocation step in elongation and is functionally homologous to bacterial EF-G. Like eIF-4D and EF-1α, EF-2 contains a unique posttranslational modification of a histidine (His-715 in mammalian EF-2 [232]) into diphthamide (2-[3-carboxyamido-3-(trimethylammonia)propyl]histidine) (297). On the basis of genetic evidence obtained from yeast cells, at least five different steps are required for the synthesis of diphthamide (36); however, this modification does not appear to be required for normal cell growth (211). The modification is required for the protein to be ADP-ribosylated by diphtheria toxin, a reaction which inactivates the protein. Inasmuch as this protein is the only cellular substrate for this modification by diphtheria toxin, [^{14}C]NAD and diphtheria toxin are often used to radiolabel the EF-2 to facilitate quantitation, because there can readily be stoichiometric modification of EF-2. The amino acid sequence of EF-2 appears to be highly conserved, showing a very high degree of identity within mammalian species (greater than 99%) (232) and reasonable homology to archaebacterial EF-2s, whereas the homology with EF-G is more limited and exists mostly in the GTP-binding domain (261). The sequence around the diphthamide residue represents the most conserved region in the molecule.

EF-3

For many, this comes as a shock. The excellent homologies between EF-1α and EF-Tu, between EF-1βγ and EF-Ts, and between EF-2 and EF-G make the existence of an additional elongation factor surprising. The comforting feature is that such a protein appears to exist only in yeasts and fungi and that its function is dependent on yeast ribosomes (130, 231, 274, 292). EF-3 has been purified to homogeneity and appears to be a single polypeptide with a molecular mass of about 125 kDa. By itself, EF-3 displays a ribosome-dependent nucleotidase which is most effective with ATP. EF-3 is about an order of magnitude more active than EF-2, which also displays a ribosome-dependent GTPase activity (292). Although EF-3 is only slightly stimulatory at high GTP concentrations (i.e., 25% at 1 mM GTP; Fig. 6 in reference 292), the stimulation of polyphenylalanine synthesis at low to physiologic concentrations of GTP can be considerable, i.e., 5- to 30-fold depending on the exact concentration of GTP (273, 292). The mechanism of how EF-3 accomplishes this is still under investigation.

RF

Unlike the bacterial system, in which there are two codon-specific release factors (RF-1 and RF-2) and another protein which enhances their activity in a GTP-dependent manner (RF-3), there appears to be only a single release factor in eukaryotes. This protein, which is functional as a dimer, requires GTP for activity (138, 283). Recently the amino acid sequence was deduced from a rabbit liver cDNA sequence which encoded 475 amino acids (160). At the time it was noted that the only proteins in the data base similar to RF were several tryptophanyl-tRNA synthetases. A more striking homology (90%) was noted subsequently, when the sequence of a mammalian tryptophanyl-tRNA synthetase was determined (83). Although this might suggest that a unique tRNA may be associated with the chain termination step, there is currently no evidence of such a tRNA. Curiously, although RF requires GTP for function, it does not contain the normal GTP consensus sequence elements (57). One assumes that the GTP specificity evolved from the ATP-binding site in the tryptophanyl-tRNA synthetases. The two-subunit structure is characteristic of tryptophanyl-tRNA synthetases although uncharacteristic of the general class 1 synthetases (45, 74).

MECHANISM OF EUKARYOTIC PROTEIN SYNTHESIS

Initiation

The overall scheme for the generation of initiation complexes is presented in Fig. 1. The first concern is to generate a substantial amount of free 40S subunits with which to begin forming initiation complexes, because under normal physiologic conditions, the formation of inactive 80S ribosomes is favored. There is, however, a small pool of free 40S and 60S subunits as a result of the equilibrium between subunits and monosomes aided by the release of free subunits at the end of the translation process. Both subunits are targets for binding proteins, which subsequently do not allow for binding of the other subunit. Most of the activity which keeps the subunits apart resides in eIF-6, which binds exclusively to the 60S subunit (235, 246). The second protein to function in this antiassociation activity is eIF-3, which binds exclusively to the 40S subunits (15, 229, 287). These two proteins combined provide for the supply of free (sometimes called "native") 40S subunits for initiation, which in fact have a sedimentation value of about 43S. The formation of this complex is assisted by the binding of eIF-4C (92).

The next step is the binding of the ternary complex of eIF-2 · GTP · Met-tRNA$_i$, which occurs in the absence of mRNA. It should be noted that the ternary complex will bind to free 40S subunits in the absence of other translation factors, but that the amount of stable 40S complex isolated is considerably reduced (15, 228). Not only is the complex of the 40S subunit and the ternary complex obtained in better yield when eIF-3 is present and the complex is isolated at 4°C, but at elevated temperature (12°C) the only ternary complex associated with 40S subunits is stoichiometric with bound eIF-3 (228). As has been shown by practically every investigator who has worked with eIF-2, the nonhydrolyzable analog of GTP (either GDPNP or GDPCP) will substitute for GTP at this point and, in fact, until subunit joining.

The next step is still a mystery, in large part because of our poor understanding of the ligand, mRNA. Under most circumstances, mRNA is associated with proteins from its start in the nucleus until its entrance into the cytoplasm. Because there is such poor understanding of how proteins influence mRNA as a possible ligand for the 43S complex, most reviews avoid the issue altogether. However, sooner or later these mRNP proteins will have to be dealt with. For optimal attachment of mRNA to the 43S complex, three initiation factors are required as well as ATP. The first protein to bind appears to be eIF-4F, and it recognizes the m^7G cap structure at the 5′ end of eukaryotic mRNA (11). This recognition is accomplished via the 24-kDa subunit of eIF-4F, which in fact can recognize this structure in the absence of the 220-kDa subunit (71, 237, 279). Several reports have indicated that in general the availability of the m^7G cap for interaction with the 24-kDa subunit of eIF-4F correlates well with the efficiency with which the mRNA is translated (86, 157). Thus this simple recognition appears to have dominant kinetic consequences.

Following the binding of eIF-4F to the cap structure of the mRNA, eIF-4B associates with eIF-4F if in fact it is not already associated with eIF-4F at the time it binds to the m^7G cap. This latter possibility is suggested by the tight association of these two proteins, which requires 0.5 M salt to effect a separation of the two (97). At this point, unwinding of the mRNA in the vicinity of the cap structure occurs (156, 234); however, additional molecules of eIF-4A and eIF-4B may be required for the ATP-dependent unwinding of more distal structures. Although eIF-4A has been characterized as an RNA helicase (as a member of the D-E-A-D box family) (166, 303), its ability to unwind mRNA secondary structure is strongly stimulated by eIF-4B (156, 245). Also, although ATP appears to be required for mRNA scanning (see below), recent experimental data suggest that the primary requirement for ATP in mRNA utilization is in mRNA unwinding (120).

With some or all of these initiation factors associated with the mRNA and at least limited unwinding of the mRNA, the next step is the binding of the mRNA · factor complex to the 43S complex (containing eIF-3 and the ternary complex). Unlike bacterial systems, which have an alignment capability for binding mRNAs, the AUG codon, and the Shine-Dalgarno sequence (100), eukaryotic mRNAs appear to lack any sequence which is specifically recognized by the ribosome. Therefore, it is inferred that the major determinant for locating the mRNA on the 40S subunit is the protein factors associated with the mRNA. Good candidates for this protein determinant are eIF-4F and eIF-4B, which both seem capable of interacting with eIF-3 (97, 103, 239). Given that eIF-4F in particular is likely to be bound only at the m^7G cap, this allows for the placement of the 5′ end of the mRNA on the 40S subunit (readers with more of an interest in the three-dimensional placement of these factors on the ribosome should consult the excellent review by Nygård and Nilsson [207]). This would be in keeping with one of the first requirements of the scanning hypothesis, i.e., that mRNAs be bound initially at their 5′ ends (140, 141). This, however, does not position the AUG codon correctly for the initiator tRNA as this codon is usually 50 to 100 bases 3′ of the m^7G cap.

To effect the correct positioning, it has been hypothesized that the 40S subunit migrates in a 5′-to-3′ direction searching for the AUG codon; this process has been termed "scanning" (140). This has posed two basic questions: what is the biochemical mechanism for scanning which experimentally seems to require ATP (141), and how is the initiation codon recognized? The first question continues to have no answer, and it has been suggested that perhaps eIF-4A, eIF-4B, and eIF-4F might provide this capability given that they participate in an mRNA-dependent ATP hydrolysis reaction. The second possibility is that this property represents an activity inherent to the 40S subunit. As will be addressed in the section on regulation, the former seems more likely. Given the ability to move the mRNA on the surface of the 40S subunit, how does the 40S subunit complex locate the initiating AUG codon? From genetic studies with *S. cerevisiae*, the answer appears to be recognition by the anticodon of the initiator tRNA (38, 39, 65). Consistent with this simple idea was the observation that more than 90% of eukaryotic mRNAs use the first AUG 3′ from the cap structure to initiate protein synthesis (145). Do the exceptions to this simple idea indicate that this is incorrect? At present I am inclined to say that the idea is valid, but our biochemical knowledge of the recognition event is too imprecise to account for the exceptions to the first-AUG rule.

There are, however, some data that may provide the start of an answer. The first was the concept that a preferred context around the AUG might ensure that the initiator tRNA used the correct AUG, and by computer search and biochemical experimentation the consensus start sequence has been determined to be A/GXXAUGG (145). The second concept is that there may be a kinetic component such that rapid scanning may pass over an AUG and only select an AUG codon when scanning is slower. This idea receives support from studies which demonstrate that RNA secondary structure can block the migration of scanning 40S subunits (144, 226) and from the general observation that by computer modeling, most eukaryotic mRNAs lack secondary structure in their 5′ untranslated region but contain extensive secondary structure in the coding region. (This observation was first made evident to me by H. O. Voorma in 1986 [299a].) This would suggest, then, that the scanning 40S subunit moves quickly through the 5′ untranslated region and slows or perhaps even stalls when the extensive secondary structure of the coding region is reached. Another element favoring this "hypothesis" is the general decline in observance of the A/GXXAUGG consensus as more eukaryotic organisms are studied, although there continues to be a preference for a purine at position −3 (35).

Once an appropriate match is made between the anticodon of the initiator tRNA and the AUG start codon, the eIF-2 molecule is poised to allow hydrolysis of the bound GTP molecule, an event triggered by eIF-5 (15, 85, 179, 229, 287, 300) and, as noted earlier, perhaps by an eIF-5 molecule complexed with eIF-4C (260, 287). This hydrolysis event causes the release of the initiation factors from the surface of the 40S subunit, and this now allows for 60S subunit joining. A curious feature of this reaction (at least the GTP hydrolysis) is that it occurs more rapidly in the presence of 60S subunits, which may indicate that the 40S and 60S subunits exist more like an opened clam, touching at a hinge point ready to close, rather than as completely free-floating entities (179, 184). This might also provide for the triggered release of eIF-6 from the 60S subunit to allow for joining to the 40S subunit complexed with Met-$tRNA_i$ and mRNA. Also consistent with this sequence is the observation that GTP hydrolysis occurs before the Met-$tRNA_i$ becomes sensitive to the action of puromycin.

In the standard bacterial system, the result of such subunit joining would be that the initiator tRNA would be correctly positioned in the P site to participate in the synthesis of the

first peptide bond. It appears that in eukaryotes some additional conformational change must occur and that it is brought about by eIF-4D as judged by the ability of 80S initiation complexes to react with puromycin (15). The nature of this reaction is poorly understood, but it does appear necessary for growth in *S. cerevisiae* (259).

As a product of 80S complex formation, eIF-2 complexed with GDP is released from the 40S subunit. Because of the slow release under physiologic conditions, protein synthesis would halt once every eIF-2 molecule had gone through the initiation cycle. To allow efficient, catalytic use of eIF-2, a second protein (eIF-2B) exists to facilitate the exchange of eIF-2-bound GDP for GTP. Two different mechanisms have been proposed to describe this nucleotide exchange, one analogous to the bacterial EF-Tu · Ts scheme (244) and one which involves a quaternary complex of eIF-2, eIF-2B, GDP, and GTP (63). Although there continues to be some controversy about which mechanism is correct, the possible existence of two nucleotide-binding sites in eIF-2 (see the section on eIF-2, above), a GTP-binding site in eIF-2B (60), and the complexity of eIF-2 relative to EF-Tu (three subunits versus one subunit) and of eIF-2B relative to EF-Ts (five subunits versus one subunit) might favor the quaternary mechanism. In contrast, with the possibility that the γ subunit is very much like EF-Tu (see the section on eIF-2, above), one would be more inclined to favor the EF-Tu · Ts scheme. Independent of which recycling mechanism is correct, the product of the recycling scheme is eIF-2 · GTP, which is now capable of binding initiator tRNA and starting another round of initiation.

Initiation of Translation—Tidbits

There are a couple of additional points which should be considered after the above general overview of the initiation pathway. The first is a listing of the characteristics which appear to be desirable for an mRNA to be efficiently recognized. These include (i) an m^7G cap structure which is readily accessible, (ii) a lack of secondary structure in the 5′ untranslated region, (iii) no AUG codons 5′ of the authentic (or desired) initiating AUG, and (iv) a 5′ untranslated region of under 100 to 150 nucleotides. Most eukaryotic mRNAs generally satisfy these ideals by having 5′ untranslated regions of 100 nucleotides or less and by having a rather low guanosine content in the 5′ untranslated region. This latter feature tends to ensure that this stretch of RNA lacks upstream AUGs and contains relatively little secondary structure. These ideal features notwithstanding, not all mRNAs are created equal. Given that there is a general limitation of the mRNA-specific initiation factors (especially eIF-4B and eIF-4F), mRNAs must compete for these limited factors; the consequence of this has been modeled mathematically (87, 170). Numerous studies by Thach's laboratory have demonstrated the validity of this mathematical model both in vitro and in vivo (22, 23, 155, 302), and, as will be observed later, these principles are at work in numerous regulatory situations.

The second tidbit is that it is quite probable that not all of the proteins which participate in the initiation process have been fully authenticated as initiation factors (i.e., have a real eIF designation). Such an example is a factor referred to as co-eIF-2C, a 94-kDa protein which enhances the formation of the ternary complex and stabilizes the ternary complex against disruption by mRNA (101). A second protein is a 67-kDa protein which associates with eIF-2 and thereby blocks the phosphorylation of the α subunit by two highly specific eIF-2 kinases, heme-controlled repressor (HCR) and double-stranded RNA-dependent protein kinase (dsI) (48). This latter protein may be more involved in regulating eIF-2 activity or may be a more integral part of the eIF-2 molecule, since it is a common contaminant of most eIF-2 preparations, even at a level of 90% purity. The other proteins currently on the likely list for factor designation are not part of the normal initiation scheme presented in Fig. 1, but rather are associated particularly with the translation of either poliovirus or encephalomyocarditis virus mRNAs (see Alternate Initiation Schemes, below). So far, two proteins of 52 and 57 kDa have been characterized (19, 20, 53, 122, 178). These are normal cellular proteins, and so one assumes that whatever their function, there will be cellular mRNAs which will also use these proteins to facilitate their translation. To my knowledge, the only viable candidate for such a cellular mRNA is the one which codes for the immunoglobulin heavy-chain-binding protein (172, 257), although a few other candidates have been suggested as SDS-gel bands indicative of host cellular proteins expressed late in poliovirus-infected cells; these could, however, also be the most efficient cellular mRNAs which manage to use the few intact eIF-4F molecules that remain. It is possible that numerous subtle effects have been missed by the assays used to characterize the "authentic" initiation factors and generate the pathway in Fig. 1. However, the very clever use of genetics, with the posing of the right question, promises to make up for these shortcomings, probably in numbers yet unimagined. It is not likely that the core of the eukaryotic mechanism is invalid, just that it is inadequate.

Alternate Initiation Schemes

In the past several years, it has become apparent that there are two alternate methods to get ribosomes to an initiating AUG codon. The first of these is reinitiation (1, 102, 133, 142, 143, 194, 225). By definition, these mRNAs must be polycistronic, having at least two open reading frames (ORFs), although in several instances the 5′-most reading frame is rather small (1, 102, 194). The suggestion is that the first initiation event occurs as presented in Fig. 1. After completion of the polypeptide chain, the 40S subunit continues to scan or move down the mRNA, although a certain percentage of the 40S subunits are lost at the termination step or during the subsequent scanning (1, 142, 143). At some point, a new ternary complex must be acquired, both to serve as the initiator tRNA and to locate the next initiating AUG. Once this occurs, presumably all of the components necessary for AUG selection and subsequent subunit joining are in place. The question again arises of how the ribosome moves. Is it the same scanning as in the initial event? If so, this would seem to favor the notion that the 40S subunit has the inherent capability to scan rather than using the mRNA-specific factors (eIF-4A, eIF-4B, and eIF-4F) and their ATPase activity. Unfortunately, the assays to test for the specific factor requirements in vitro will be complicated by the presence of factors necessary for the first initiation event. The obvious substrate would be where a ribosome is stalled in the middle of an ORF by amino acid limitation, isolated by sucrose gradients, and then used to translate the second ORF. This will not be trivial.

The second rare initiation event is internal initiation, which has often been characterized as cap-independent initiation. This type of translation was first noted for the picornaviruses, which lack a 5′ m^7G cap structure. Simply put, the 43S complex (with eIF-3, eIF-4C, and the ternary

complex) binds to a portion of the mRNA distant from the 5′ end and then scans, if necessary, to locate the initiating AUG codon. What is necessary from the mRNA to allow binding is uncertain. The simplest idea would suggest a lack of secondary structure in the RNA which would readily allow binding of translation factors eIF-4A and eIF-4B, and this might be enough to provide the protein determinant for binding of the mRNA (3). However, most studies have found that eIF-4F is also necessary for optimal translation (3, 9, 30, 154). Clearly, more work is necessary to resolve this question and, in particular, the question whether additional proteins may be required for this process depending on the mRNA (i.e., similar to the 52- and 57-kDa proteins cited above) (19, 53, 178). That this process is truly an internal initiation was first demonstrated by using a bicistronic mRNA with an intercistronic region that represented the 5′ untranslated region of poliovirus mRNA (227). Internal initiation of viral and synthetic construct mRNAs has subsequently been confirmed by many researchers. The task is now to determine the biochemical events of this process.

Elongation Cycle

With the initiator tRNA firmly entrenched in the P site of the 80S ribosome, the repetitive cycle for the codon-directed addition of aminoacyl-tRNAs is set to begin. As noted above, there is considerable homology to the bacterial system at the level of the primary sequence for EF-1α (versus EF-Tu) and EF-2 (versus EF-G) and a strong functional similarity of EF-1βγ (versus EF-Ts). Thus much of this presentation will be focused along the better-established bacterial lines including the interesting new half-site model proposed by Moazed and Noller (188), although even this has a proposed alternative (205). Having bound GTP and an aminoacyl-tRNA, EF-1α directs the binding of the aminoacyl-tRNA in a codon-dependent manner. By the half-site model, this is envisioned to first reflect a dominant interaction of the anticodon with the codon in the A site, presumably guided by an EF-1α–ribosome interaction. After the correct match has been made, some signal triggers the hydrolysis of GTP, which leads to the release of EF-1α · GDP and placement of the aminoacyl-tRNA in the A site, as illustrated in Fig. 2. The existence of such an aminoacyl-tRNA in the A site is very short lived in the presence of the aminoacyl-tRNA (or later peptidyl-tRNA) in the P site, and the one "enzymatic" activity of the ribosome is used, i.e., the formation of a peptide bond via the peptidyl transferase center of the large subunit, occurs very quickly. In this reaction, there is an apparent nucleophilic attack by the α-amino group of the aminoacyl-tRNA in the A site on the carbonyl of the activated ester linkage of the aminoacyl-tRNA (or peptidyl-tRNA) in the P site. This leads to the transfer of the initiator methionine (or peptide) from the tRNA in the P site to the aminoacyl-tRNA in the A site.

In keeping with the half-site model, the 3′ end of the new peptidyl-tRNA is switched to the P site while the anticodon remains in the A site. The 3′ end of the deacylated tRNA is shifted to the E site while its anticodon does not move. The consequence of this action is that the growing polypeptide chain elongates but does not move, which is consistent with biophysical data (209). The next step, translocation, is accomplished by EF-2 in a GTP-dependent manner. This process causes the movement of the mRNA by three nucleotides, i.e., one codon, so that a new codon exists in the A site. This also means that the anticodons of the unacylated tRNA and new peptidyl-tRNA are shifted, placing the unacylated tRNA fully in the E site and the peptidyl-tRNA fully in the P site. As might be appreciated, the sites on the ribosome which interact with the elongation factors EF-1α and EF-2 overlap, and for readers who do not keep track of the ribosomal protein locations, this is nicely shown in three dimensions in Fig. 8 of Nygård and Nilsson (207).

At this point the ribosome is prepared to undergo the next cycle of elongation. As may be obvious, most of the energy required for protein synthesis is used during the elongation cycle and is essentially two high-energy phosphates used per cycle (EF-1α and EF-2) and two high-energy phosphates used to generate each aminoacyl-tRNA (ATP + AA + tRNA → AMP + PP_i + AA-tRNA with the PP_i usually being split into two P_i molecules). Thus the formation of each peptide bond costs four high-energy phosphates.

Peptide Chain Termination

When the peptide chain has been completed, it comes to one of three stop codons, UAA, UGA, UAG. Unlike bacterial systems, which have two proteins with codon-specific termination recognition, mammalian systems have but a single factor involved in termination (referred to as release factor [RF]). In cell-free assays, this factor shows a marked requirement for four nucleotides (the stop codon plus one), whereas in the equivalent assay using the bacterial RFs, a trinucleotide was sufficient (236, 283). This requirement may exist in vivo also, as an analysis of many eukaryotic stop signals shows a very specific bias for the nucleotide just following the stop codon (24). With bound GTP, RF recognizes the stop codon and induces the hydrolysis of the aminoacyl linkage concomitant with the hydrolysis of GTP and subsequent release of the peptide and RF · GDP.

The action of RF is necessary to cause efficient termination and thus allow the recycling of the ribosomes and mRNA for another round of translation. In addition, stalled ribosomes may shift the reading frame to continue synthesis, which would lead to an aberrant product (frayed carboxy terminus). It should be noted that besides causing an effective termination event, RF may also be responsible for deciding what percentage of the 40S subunits may remain attached to the mRNA and thus allow for reinitiation. Although there is no direct proof that this capability resides in RF, mutational analysis in the GCN4 system in *S. cerevisiae* would support this hypothesis (see below).

REGULATION OF TRANSLATION

The discussion of the control of protein synthesis will focus primarily on the regulation of the activity of the translation factors, although some attention will also be given to a few examples of regulation at the level of available mRNA. It should be said that there is an exceptionally large body of literature which cites different biological systems as being under translational control. This is usually evident from Northern (RNA) blots, which show a fixed level of mRNA and protein measurement of uptake of radioactivity into a particular band in an SDS-gel or increase in reaction with an antibody as directed by Western immunoblots. Although this would normally be the hallmark for translational control, authenticated studies would more carefully assess polysome loading and half-transit time as well (see, e.g., reference 193). For considerably more systems than will be reviewed in this article, the reader is directed to three relatively recent books which focus on translational control (117a, 283a, 286a).

Control by Protein Phosphorylation

The first discussion will focus on control of translation at the level of protein synthesis initiation. Although they are not the exclusive elements, most regulation relates to the availability of the ternary complex (Met-tRNA$_i$ · GTP · eIF-2) or of "activated" mRNA which can bind to the 43S preinitiation complex (Fig. 1). To a first approximation, reduction in the level of the ternary complex does not disturb the ratio of proteins synthesized relative to each other, but causes only a percent reduction in all of them. Regulation of activated mRNA, however, is generally mRNA selective, leading to the most drastic reduction for mRNAs which compete poorly for the mRNA-specific translation factors.

The first target of regulation to be discussed is eIF-2. As noted in the early discussion on eIF-2, it has a 100-fold preference for GDP and, with a K_d for GDP of 10^{-8} M, a very slow off rate. These characteristics necessitate an exchange factor, eIF-2B. Although the actual mechanism of nucleotide exchange has not been unequivocally determined (63, 244), the regulation of ternary complex has, and this is through the level of exchange factor activity. The classic examples of this type of regulation are the activation of HCR, a heme-sensitive protein kinase, and dsI, a kinase which requires double-stranded RNA for activity (37, 80, 116, 163). These kinases lead to the phosphorylation of the α subunit of eIF-2 at Ser-51 (41), although evidence has been presented for phosphorylation at Ser-48 as well (146, 150). That these sites are functional in vivo was shown by expression in tissue culture cells of the α subunit carrying either Ala (no phosphorylation possible) or Asp (a mimic of permanent phosphorylation) in place of the normal Ser (49, 132). The mechanism derived to explain the effect of the phosphorylation of the α subunit is that the phosphorylated eIF-2 · GDP complex released during the initiation of protein synthesis binds to the recycling protein, eIF-2B. The complex is stable, but cannot exchange the bound GDP for GTP. As a consequence, the eIF-2, which is usually in excess of eIF-2B by a factor of 2 to 10, ties up the eIF-2B, thus depleting the system of the needed recycling activity. This model is consistent with the general observation that much less than stoichiometric phosphorylation of the α subunit of eIF-2 is necessary to shut off protein synthesis as the remaining eIF-2 accumulates as eIF-2 · GDP. And, as might be expected, although the classic examples of eIF-2α phosphorylation result from activation of protein kinases, inactivation of the appropriate phosphatase also leads to elevated levels of phosphorylated eIF-2 (135).

Regulation of the ternary complex as evidenced by eIF-2α phosphorylation has been associated with a number of different physiologic states other than heme deficiency and viral infection (which causes the generation of double-stranded RNA) as described above, including heat shock, the presence of heavy metals, and deprivation of serum, amino acids, glucose, or insulin (66, 67, 117, 262, 286). However, it is possible that the activity of eIF-2B is regulated. This possibility is based on the observations that eIF-2B binds NADPH and is inhibited by $NADP^+$ (61), that eIF-2B binds GTP and two of the subunits of eIF-2B bind ATP (60), and that phosphorylation of the 82-kDa subunit of eIF-2B by casein kinase II can activate the in vitro exchange activity fivefold (62). Given that the γ subunit of eIF-2 appears similar to EF-Tu, it is surprising that there is such a disparity in their respective nucleotide exchange factors (EF-Ts, 30 kDa; eIF-2B, 82, 67, 58, 39, and 26 kDa). Although part of this may be associated with the "non-EF-Tu-like" exchange mechanism (63), it is also likely to be a sign that the exchange factor itself may have considerably more complex regulatory signals than are evidenced by eIF-2α phosphorylation. This allows for regulation via a different protein (i.e., eIF-2B directly) and thus increases the opportunity to fine tune regulation of ternary complex formation.

The second target for regulation of activity is formed by the mRNA-specific translation factors, eIF-4A, eIF-4B, and eIF-4F. There has been no report on the posttranslational modification of eIF-4A or on the acute regulation of levels of eIF-4A protein. That the two isozymes of eIF-4A are expressed in a tissue-specific manner (204) may yield tissues with slightly different translational capacities, but the levels are not known to be regulated within a specific tissue. On the other hand, both eIF-4B and eIF-4F are phosphorylated proteins (109, 110, 288). Under numerous conditions, eIF-4B has been shown to be phosphorylated, and there is an excellent correlation between the level of protein synthetic activity and eIF-4B phosphorylation, with the highest levels associated with the most extensively phosphorylated eIF-4B (67, 70, 109, 190, 191). Isoelectric focusing studies show that eIF-4B is multiply phosphorylated (perhaps as many as 10 phosphates). The amino acid sequence indicates 10 sites each for casein kinase II and protein kinase C by using consensus site analysis (185), but more experiments are needed to determine whether any or all of these sites are used.

In a similar manner, extensive phosphorylation of eIF-4F is also correlated with enhanced protein synthetic activity. Characterization of the multiple phosphorylation sites on the 220-kDa subunit has been difficult because of the size of the peptide and its sensitivity to protease. However, unlike eIF-4B, it has been demonstrated in vitro that the fully phosphorylated eIF-4F is about five times more active than the unphosphorylated eIF-4F (189). Where it has been examined, it would appear that the phosphorylation of eIF-4F and eIF-4B (and ribosomal protein S6 as well) is coordinately regulated, with all factors displaying enhanced phosphorylation with enhanced protein synthetic activity (67, 109, 110, 190, 191, 288).

However, considerably more work has been done with the small (24-kDa) subunit of eIF-4F, in large part owing to the isolation of cDNAs for the yeast and mammalian proteins (4, 5, 248). This subunit undergoes phosphorylation (at Ser-53 [250]) under the same types of conditions that lead to enhanced phosphorylation of the 220-kDa subunit of eIF-4F and eIF-4B (67, 68, 70, 190, 191, 217). In a study analogous to those described for the α subunit of eIF-2, Ser-53 was mutated to an Ala, and the resulting mutant 24-kDa peptide was incapable of participating in the formation of initiation complexes, suggesting an absolute requirement for phosphorylation of Ser-53 for activity (125).

An unexpected finding about the 24-kDa subunit was that its overexpression would cause malignant transformation (159), and this effect could also be accomplished by the microinjection of either the 24-kDa subunit or eIF-4F (276). Although it may be difficult to compare different cells, in studies which quantitated the levels of all of the subunits of eIF-4F the most limiting component was the 24-kDa subunit (about half the level of the 220-kDa subunit) and the complete factor, eIF-4F, appeared to be limiting in cells (26, 68). The conclusion reached is that by increasing the levels of active eIF-4F, poorly translated mRNAs were now overexpressed, and of these mRNAs, the proto-oncogene mRNAs would be likely candidates for such increased expression

given that they tend to be poorly translated and have relatively long 5′ untranslated regions and often short ORFs 5′ of the initiating AUG (159). However, no elevated synthesis of proteins such as c-*sis*, *lck*, or c-*myc* has yet been reported as a result of this overexpression. This elevation of eIF-4F activity and the enhanced translation of poor mRNAs are predicted from the mathematical model (87).

As noted above, it is likely that different tissues contain different levels of the many translation factors. A very interesting report would appear to indicate an even greater level of complexity, namely that the level of one polypeptide may influence that of another. The particular study indicated that reduced expression of the 24-kDa subunit of eIF-4F (achieved by the use of antisense RNA) was associated with a reduced level of the 220-kDa subunit of eIF-4F (52). Whether this effect is general (relating to all factors), more specific (relating only to subunits of translation factor aggregates), or a single isolated example is not known and requires further study. However, the regulation of factor peptide levels combined with the many factor phosphorylations already known would allow for exceptionally complex global control of translation.

Phosphorylation and dephosphorylation have been demonstrated to effect translation at the level of ternary complex formation and mRNA binding both in vivo and in vitro for eIF-2 and eIF-4F. At the same time, correlated with this has been the phosphorylation of eIF-4B and ribosomal protein S6. Although not as many studies have been performed, both eIF-3 and eIF-5 are more highly phosphorylated under conditions of enhanced protein synthesis (67, 70, 109, 110, 288). In total, for the process of initiation, phosphorylation of the α subunit of eIF-2 causes inhibition whereas all other phosphorylations either cause or are correlated with enhanced protein synthesis. There currently appear to be 30 to 40 phosphorylation sites involved in the stimulation of translation, and so it is clear that if each contributes slightly to enhanced translation in vivo, an exceptional level of fine tuning is possible.

Although not as well characterized as the circumstances that lead to the covalent modification of initiation factors, EF-1 and EF-2 appear to undergo posttranslational modification that regulates their activity or that correlates well with changes in protein synthetic rate. For one of the longest standing, methylation of EF-1α during germination has been correlated with increased levels of activity in *M. racemosus* (82), although even the most recent report has failed to show where EF-1α activity might be regulated (269). EF-1α has also been reported to be phosphorylated (51), but it is likely that this "phosphorylation" represents the addition of glycerylphosphorylethanolamine (55); to date there is no evidence that this modification affects activity in vivo, and it does not affect activity in vitro (34a). In contrast is the more recent observation of EF-1γ phosphorylation of Ser-89, decreasing the ability of the EF-1βγ complex to catalyze the nucleotide exchange reaction (EF-1α · GDP + GTP → EF-1α · GTP + GDP) (123). Although these authors (and others [215]) cite casein kinase II as the likely kinase to effect this modification in *A. salina*, another group has identified the $p34^{cdc2}$ kinase as phosphorylating EF-1β and EF-1γ in vivo in *X. laevis* (14). Although the activity of the phosphorylated EF-1βγ was not tested, it did correlate with the changes that accompany the expression of the $p34^{cdc2}$ protein kinase activity.

A different set of experiments has indicated that EF-1 activity is enhanced by phosphorylation in vivo with phorbol ester or in vitro phosphorylation with protein kinase C (298, 299). The predominant phosphorylation in this instance is on the β and δ subunits. Thus it appears that the activity of the recycling protein EF-1βγδ can be regulated either positively or negatively depending on the actual kinase responsible for the phosphate addition.

As was noted for EF-1α, EF-2 also undergoes posttranslational modification. The conversion of His-715 to diphthamide was noted above, and this modification is not required for activity. However, two different modifications do inhibit EF-2 activity. These include mono-ADP-ribosylation and phosphorylation. The mono-ADP-ribosylation was originally reported as catalyzed by diphtheria toxin in the presence of NAD (114), but more recent reports suggest that cells also contain an enzyme for mono-ADP-ribosylation of EF-2 (161, 175, 271). The net effect is to decrease protein synthesis as the modified EF-2 appears to bind to ribosomes but is ineffective in promoting translocation (50). Thus, not only is there a decrease in the percentage of active EF-2 molecules, but also the ribosomes are inhibited when the inactive EF-2 is bound.

EF-2 is also phosphorylated (202, 206), the original report citing it as the major substrate for calcium/calmodulin-dependent protein kinase III, a protein kinase whose activity can be regulated by phosphorylation (202). The sites of phosphorylation appear to be the threonine residues at positions 56 and 58 in the mature protein (230). It has also been observed that EF-2 can be both phosphorylated and mono-ADP-ribosylated, yielding a variety of inactivated forms of EF-2 (175). Enzymatic studies of the phosphorylated EF-2 indicate that although it appears to have many of the characteristic activities of EF-2, its major defect is its poorer binding capacity to pretranslocation ribosomes, which is reduced by a factor of 10 to 100 (32). And as was noted for eIF-2, the level of phosphorylated EF-2 can be regulated by the level of phosphatases, in particular the type 2A protein phosphatase, whose activity is induced by treatment with phorbol ester (99).

The above examples are likely to represent only a portion of the possible regulatory phenomena for the translation factors. A large number probably will emerge with the further development of yeast genetics in the study of translational control. Second, given that a particular site undergoes phosphorylation and dephosphorylation, no real effort has been made to exhaustively characterize the possible or probable kinases and phosphatases involved. Finally, the total number of covalently modified sites is already large (>40 sites), so that very complex yet fine-tuned patterns of control are possible. As noted above, in general one expects only the modifications of the mRNA-specific factors to dramatically alter the ratios of protein products made. The topics to be developed below are examples of specific regulation of different mRNAs. These are not intended to represent all possible modes of regulation, but rather to serve as examples. It may then be possible for the reader to piece together single or combinational events that might be involved in the ever-increasing examples of translational control.

Control of Translation of Specific mRNAs

The examples of translational control have one feature in common, autoregulation. The three examples chosen are the synthesis of ferritin (and related proteins), tubulin, and GCN4. The ferritin story is especially intriguing because it is the only specific example of how an mRNA is actively prevented from participating in protein synthesis, a general

phenomenon of "stored mRNAs" usually found in egg or seed stages of organism development. The system seems rather simple: a sequence of bases, 35 nucleotides in length, fold to form a specific stem-loop structure (see Fig. 3 in reference 34) that is recognized by an 87-kDa protein (34, 104, 242, 301). Proteins which are needed in larger amounts in the presence of iron have mRNAs which contain the 35-nucleotide iron-responsive element (IRE) in the 5′ untranslated region (ferritin, aminolevulinic acid [ALA] synthase [181]). The transferrin receptor which is needed in larger amounts in the absence of iron is expressed from an mRNA which contains several copies of the IRE in the 3′ untranslated region (196, 197). The following plan seems to be in effect for these mRNAs. The transcription rate for the mRNAs is roughly constant, and so the regulation of protein production is posttranscriptional. In the absence of iron, the repressor protein binds to all the mRNAs. For mRNAs which contain the IREs in the 5′ untranslated region, this blocks translation of the mRNA and thus little or no protein is made (ferritin, ALA synthase). At the same time, binding of the 87-kDa repressor protein to the IREs in the 3′ untranslated region causes a stabilization of the mRNA, and as a result of the change in half-life, the mRNA levels rise, thereby increasing protein production (transferrin receptor). As iron levels increase, the ability of the repressor protein to bind to the IREs is weakened, and this reverses the above pattern. Now ferritin and ALA synthase are made efficiently, while the mRNA for the transferrin receptor is less stable and turns over more quickly and the mRNA level falls.

This entire scheme makes sense in the larger picture of iron metabolism. Ferritin is the iron storage protein and ALA synthase is the key enzyme in heme biosynthesis. When iron levels are high, ferritin is necessary to package the excess iron in a nontoxic form and heme biosynthesis is favored by the elevation of ALA synthase. When iron levels are low, the increased levels of the transferrin receptor facilitate the uptake of the carrier of molecular iron, transferrin. This scheme to balance so many proteins through a common protein and *cis*-acting element reflect that normal dietary iron levels can be quite low (we didn't always have vitamins with iron supplements), but should a high level of dietary iron be encountered, this potentially toxic element would be safely stored. It is still not known how the repressor protein is regulated. Different schools of thought suggest that either heme (165) or chelatable iron (73) is the regulator of repressor protein binding. The recent observation that the repressor protein shows extensive homology to the iron-sulfur protein aconitase (107, 241) would seem to favor iron as the regulator, although conventional wisdom would favor the end product of the iron pathway, heme, as the regulator. It has also been suggested that both might be regulators and which of the two predominates would depend on the cell type (73). Time will tell.

The second autoregulated system is that for β-tubulin, which, along with α-tubulin, is the major constituent of microtubules. Although free α- and β-tubulin are in constant equilibrium with polymerized tubulins in microtubules, cellular events influence the equilibrium. As a sensing mechanism, the concentration of free heterodimers (containing one α and one β subunit) regulates the stability of the mRNAs. The current mechanism to account for this phenomenon suggests that the first four amino acids (MREI) are the key to regulating stability (84, 311). By computer search, this sequence is unique to β-tubulin. On the basis of experimental data, the model proposes that as the β-tubulin chain emerges from the ribosome, it senses the concentration of free heterodimers by direct binding (favored by high concentrations). If binding to the emerging β-tubulin chain occurs, a nuclease which is resident on the ribosome is activated and the β-tubulin mRNA is degraded (see Fig. 5 in reference 84). If the heterodimer concentration is low, binding to the nascent chain does not occur, the mRNA remains intact, and continued synthesis of β-tubulin ensues. This mechanism of regulation has not been generally observed and therefore could relate to only a few mRNAs. On the other hand, this may also be the prototype for the numerous examples emerging in which mRNA instability requires translation of the mRNA (12, 93, 148, 168, 196, 223, 267, 272).

The last autoregulatory system to be discussed is the expression of the GCN4 protein in *S. cerevisiae*; this protein is a transcription activator for amino acid biosynthetic enzymes in *S. cerevisiae*. The general system is that in the absence of amino acids, GCN4 expression increases without an increase in mRNA content. Once amino acid levels rise, GCN4 expression is reduced, again with no apparent change in mRNA levels. The analyses of this system by the Hinnebusch and the Thireos laboratories have been instrumental in piecing together the story outlined below (1, 112, 149, 186, 194, 243, 289, 305). For simplicity, the apparent final model is presented, but interested readers are encouraged to go back to the original literature to see how the problem unfolded. The GCN4 mRNA contains a 590-base 5′ untranslated region with four small ORFs (3 or 4 amino acids in length) beginning at about 230, 300, 415, and 440 nucleotides 3′ to the m^7G cap (referred to as ORF-1, ORF-2, ORF-3, and ORF-4, respectively). Initiation begins in the usual cap-dependent manner, and the ribosomes scan until they initiate at either ORF-1 or ORF-2. Following termination, the 40S subunit continues scanning in a 5′-to-3′ direction. However, in the absence of the ternary complex (eIF-2 · GTP · Met-$tRNA_i$), recognition of future AUG codons is not possible. Therefore, to reinitiate protein synthesis, the scanning 40S subunit must acquire a new ternary complex, and when it does so, it is fully ready to reinitiate. Under conditions of high levels of amino acids, the ternary complex is acquired quickly and a second round of initiation occurs at ORF-3 or ORF-4. If this happens, the scanning 40S subunit either falls off the mRNA or does not recover in time and consequently bypasses the AUG codon for the GCN4 ORF. On the other hand, if the ternary complex is acquired slowly, as is the case during amino acid starvation, the scanning ribosome bypasses ORF-3 and ORF-4 but recovers a ternary complex in time to initiate protein synthesis at the initiating AUG for GCN4.

It should be noted that this mechanism works because reinitiation is generally not an efficient process and a certain fraction of ribosomes are lost at each termination event. Second, this system is not designed for optimal expression of GCN4 (which can be 10- to 20-fold greater in the absence of the four ORFs [194]) but, rather, for the regulated expression of GCN4, which fluctuates about eightfold between starvation and nonstarvation conditions. The key element in the above scheme is regulating the rapidity of how a ternary complex might reassociate with the scanning ribosome. The answer here is borrowed from the mammalian system until the details are fully worked out. The controlling protein appears to be GCN2, a protein kinase with a His-tRNA synthetase-like carboxy terminus (305). Presumably, GCN2 senses uncharged tRNAs (149, 305), and this activates the protein kinase activity of GCN2. GCN2 then causes the phosphorylation of eIF-2α (56), which results in a decrease

in the nucleotide exchange activity of eIF-2B, and consequently the pool size of the ternary complex is diminished. The suggestion that GCN2 recognizes all species of uncharged tRNAs is consistent with the observation that starvation for any single amino acid will lead to enhanced GCN4 expression.

The main puzzle remaining from the simple description above is to account for the observation that the ORFs are not equivalent, in particular that ORF-3 and ORF-4 are much stronger down-regulators than are ORF-1 or ORF-2. The answer here may lie in the unique character of the termination event that occurs with ORF-3 or ORF-4. The role of the RF here and its influence on the percentage of 40S subunits that continue to scan the mRNA are likely to provide the answer. Given that a clone exists for the eukaryotic RF (160), it is likely that the yeast protein will be identified shortly and then be examined in this regulatory system.

As may have been noticed, although the reference to the above three examples was to autoregulatory control, in the strictest prokaryotic sense only the β-tubulin example counts, the others having several intermediate steps involved between the translation product and its effector of regulation. Similar types of regulation have also been observed for ornithine aminotransferase (79, 193) ornithine decarboxylase (129, 308), and the gene product of *CPA1* (306), and it is anticipated that more examples exist. The only other direct autoregulatory system that I am aware of is that for the poly(A)-binding protein (PABP), which contains an A-rich sequence in its 5′ untranslated region and which could serve to recruit the PABP and shut off translation when sufficient PABP had been made to cover the 3′ poly(A) tails on mRNAs (251).

There are numerous examples of translational control which will not be covered in this review owing to an incomplete description of the underlying mechanism (heat shock mRNAs, ribosomal protein mRNAs, maternal or stored mRNAs, hormonally regulated mRNAs, etc.). In almost all instances in which these genes have been studied, a nucleotide sequence has been responsible and, as such, has been used in chimeric constructs to evaluate the nucleotide sequence elements responsible. In most cases, the sequence element has been in the 5′ untranslated region. It is not clear how many of these situations involve a regulating protein (as in the ferritin system) or in what instances modulation of translation factor activities are involved. Proteins that are naturally poor mRNAs will usually see the greatest reduction when competition is accentuated by diminishing levels of activity for the mRNA-specific proteins, as tends to occur during development or the cell cycle.

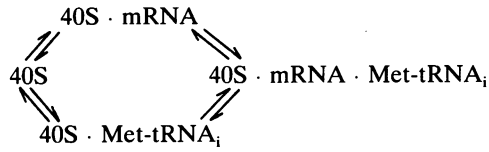
LOOSE ENDS

For those of you who have gotten this far and are still hungry for more, my heartfelt thanks. The above description of the translation factors, the mechanism of protein synthesis, and a few aspects of regulation represent most of the available data, but a number of observations were not included because they were not consistent with the model presented. After 20 years of working in the translation area, it has been my feeling that practically all of the experiments published can be repeated, although some more easily than others. Second, almost never have the data been wrong, but rather the interpretation of the data has been incorrect, occasionally, but not often, for lack of the appropriate control. Therefore, these loose bits tend to drive new experimentation to achieve a satisfactory answer, which may mean the model is inaccurate or the experiments were not complete. Finally, it should be understood that a model is by definition something unfinished that exists to facilitate the planning of new experimentation to come ever closer to the truth. With this in mind, the problems begin.

The protein that causes most of the problems is eIF-2, and these problems relate to the binding of nucleotides and RNA (see the above characterization of eIF-2). Assuming that the EF-Tu-like protein identified by Ernie Hannig is in fact the γ subunit of eIF-2, then eIF-2 minimally contains a subunit (γ) capable of binding GTP and the initiator tRNA and a subunit (β) presumably capable of binding RNA via its putative zinc finger. Second, affinity-labeling studies have consistently implicated the β subunit in cross-linking to nucleotides ATP or GTP, although usually GDP (or its derivatives) was no better at labeling or protecting, as would be expected from the reported 100-fold preference for GDP. Thus, it would appear that there could be two sites on eIF-2 for binding nucleotide and two sites on eIF-2 for binding nucleic acid, one of which is likely to be limited to the initiator tRNA.

The next observations are a direct offshoot of the above and were based on the interaction of eIF-2 with mRNA (89, 128, 296) and the effect of eIF-2 on AUG selection (47). It is probably not surprising that eIF-2 might bind RNA, given the high salt concentration necessary to elute eIF-2 from phosphocellulose, the basic pI of the γ subunit, and the three oligolysine tracts in the β subunit. Specificity is observed at the level of the initiator tRNA (nonacylated $tRNA_i^{Met}$ and nonacylated and acylated tRNAs do not compete), and apparent specificity between mRNAs has been reported (127, 128, 240). More than this, the binding of eIF-2 to internal sites near the AUG codon was indicated (89, 127). However, it has not been shown whether the recognition of the mRNA is more sequence specific or structure specific. One possible alternative is that the RNA-binding site might in fact reflect the use by eIF-2 of rRNA as a portion of its recognition of the correct binding site on the ribosome. This possibility is given additional weight by the observation that eIF-2 will bind to ribosomes in the absence of GTP or initiator tRNA if complex formation is assayed by gel filtration (but not if isolated by sucrose gradients [115a]). Another possibility is that eIF-2 must sense the presence of the mRNA to allow GTP hydrolysis and subsequent release of eIF-2 and eIF-3 from the 40S subunit, a process to be followed by subunit joining. A third possibility is that eIF-2 binds mRNA to the 40S subunit and then binds the initiator tRNA. This third suggestion would fit with the observation that eIF-2 can bind only one nucleic acid at a time, either the initiator tRNA or mRNA. As yet there is no model which reconciles all of the above data and the extensive literature on the mRNA-specific factors, eIF-4A, eIF-4B, and eIF-4F, and their role in mRNA selection and utilization.

An interesting element in one of the above reports (47) is whether the ternary complex might bind to the ribosome after the mRNA had bound. Stimulation of Met-$tRNA_i$ binding by AUG or a nearly absolute requirement for AUG to form 40S complexes has been reported (46, 228). Those examples leave open the possibility that the mRNA is bound before eIF-2. This is clearly the case for reinitiation, and the role of eIF-2 in this process appears proven. Second, the use of poly(U) as an artificial template for elongation assays indicates that mRNA can functionally attach to ribosomes without mRNA-specific factors. Thus, it is more than likely to be a case of relative kinetic rates (see below):



As might be imagined, the stabilities of the intermediate complexes (either 40S · mRNA or 40S · Met-tRNA$_i$) could be sufficiently different that only one (usually 40S · Met-tRNA$_i$) is recovered during sucrose gradient analysis. In addition, the presence of limiting or saturating levels of any of the other initiation factors may influence the choice of pathway. A third area of concern relates to many of the translation factors, but especially the initiation factors, and that is their tendency to form complexes with one another or with ribosomes. From the long view, it makes sense that factors be compatible with sharing space on the surface of the 40S subunit and, in many instances, functionally interacting with one another. However, in the short term, it makes it less obvious what the "single" pathway for initiation is, if, indeed, there is only a single pathway (see, e.g., reference 101). A simple example is the effect of eIF-3 on the ternary complex, where under conditions of low concentration, eIF-3 can stimulate ternary complex formation or methionylpuromycin synthesis. Possible equilibria are indicated below:

A. eIF-2 + GTP + Met-tRNA$_i$ $\rightleftharpoons$ 3°
B. 3° + eIF-3 $\rightleftharpoons$ eIF-3 · 3°
C. 3° + 40S $\rightleftharpoons$ 3° · 40S
D. eIF-3 + 40S $\rightleftharpoons$ eIF-3 · 40S
E. eIF-3 · 3° + 40S $\rightleftharpoons$ eIF-3 · 3° · 40S
F. eIF-3 · 40S + 3° $\rightleftharpoons$ eIF-3 · 3° · 40S

The pathway A $\rightarrow$ C $\rightarrow$ F is illustrated in Fig. 1. As noted above, other translation factors could alter the kinetic pathway chosen for binding the ternary complex to the 40S subunit. Clearly, more sophisticated kinetic studies are required to work this out.

Yet another line of experiments has questioned the validity of the flow scheme in Fig. 1; although the discussion will focus on eIF-2, concern about the other factors, especially the multisubunit proteins, is also appropriate. This altered line of experimentation is to use intact reticulocyte lysates, allow some reaction to occur, and then resolve ribosomal complexes by sucrose gradients. Individual factors and peptides are then identified by SDS-gel electrophoresis followed by Western blot analysis. Such analyses for eIF-2 suggest that the product of the initiation pathway, eIF-2 · GDP, binds to the 60S subunit and that when the phosphorylated form of eIF-2 (α-PO$_4$) is bound, this prevents the 60S subunit from joining (98, 284). Here and elsewhere, one would assume that if this occurs, the "clamlike" ribosome may be a better model than subunits which completely dissociate and then rejoin only when all factors are off the surface of the 40S subunit (otherwise the addition of exogenous 60S subunits should restore 80S complex formation). It is assumed that in the clamlike ribosome, there is a portion of the 40S and 60S subunit that is capable of interacting to provide a weak hinge that could hold the subunits in close proximity and yet allow for the surface of the 40S subunit to acquire the necessary factors, Met-tRNA$_i$ and mRNA. This might also provide for a mechanism to drive the release of eIF-6 from the 60S subunit. This type of interpretation does not challenge the model in Fig. 1 as much as it enlarges upon it, providing considerably more detail.

Similar studies of this type with antibodies to other factors are also likely to enlarge our picture of the detailed steps in translation, especially the initiation process. This consideration reflects the very considerable difference between the partial assays or reconstituted systems, which tend to be rather inactive relative to the in vivo rate of protein synthesis. In addition, almost all of the translation factors will pellet with ribosomes (eIF-4D, EF-1α, and EF-2 are more prominent in a 100,000 $\times$ g supernatant), yet few are associated with subunits or polysomes in reconstituted systems. One suspects that a number of proteins, perhaps even some ribosomal proteins released by washing with 0.5 M KCl, enhance the stickiness of ribosomal particles and that during purification these are lost. Thus progress in the mechanism of translation will in the future represent more of a mix of partial assays with purified components, the use of inhibitors and antibodies to study complete lysates, and, of course, genetics (in *S. cerevisiae*) or molecular genetics in cell cultures.

The next problem child is eIF-4F. Having been part of the discovery team, I feel some obligation to protect the sanctity of the holy three-subunit form (220, 46, and 24 kDa). At the same time, there are disquieting reports of sometimes less than the tripartite form being functional and, with shock and dismay, a small bit even from my laboratory. The original isolation of the protein that binds the m^{7}G cap of mRNAs was in fact an isolation of just the 24-kDa protein (279). As noted above, the two-subunit form (220 and 24 kDa) appears to function in reconstituted systems (27, 30, 78, 234). In vitro studies show that the in situ-synthesized 24-kDa subunit can enter into an eIF-4F complex (113, 125) and that this entry appears to require phosphorylation of the 24-kDa subunit (125, 152, 249). The amount of the 24-kDa subunit in reticulocyte lysates and HeLa cells is limiting relative to the other subunits of eIF-4F, and it is present at about 0.02 to 0.05 copy per ribosome (a concentration of about 10 to 30 nM) (68, 237). As noted above, eIF-4F will form a binary complex with any of the following: ribosomes, mRNA, eIF-3, and eIF-4B. From the above observations and data discussed previously, it seems quite likely that the subunits of eIF-4F disassemble and reassemble. What is not clear is how much of this reflects normal equilibrium equilibration (A + B $\rightleftharpoons$ AB) and whether some of the exchange is mechanistically driven (A + B $\rightleftharpoons$ AB $\overset{\text{ATP}}{\underset{\text{ADP}}{\rightleftharpoons}}$ A + B) for particular functions in binding mRNA to 40S subunits. As with eIF-2, further study is required to incorporate all of the above observations into a single flow scheme.

A third element of mystery is the common 3′ end to eukaryotic mRNAs, the poly(A) tail and the protein that binds to it, PABP. This single topic has been reviewed extensively in a recent article (121), and only some of that discussion is presented here. As accumulated nicely in Table 1 of Jackson and Standart (121), there is overwhelming evidence that in vivo the presence or increased length of a poly(A) tail yields an mRNA with increased translational efficiency. However, this addresses the phenomenon, not the mechanism. It is also unknown whether the poly(A) tail enhances the first and all subsequent initiation events or whether it specifically enhances the reinitiation events (recycling of ribosomes which have just terminated back to initiate at the 5′ end). This latter possibility is clearly raised because the kinetics of polysome formation do not appear straightforward. In particular, given limiting mRNA, one would expect that just after one transit time (the time required to synthesize the complete polypeptide chain), the mRNA would have a full complement of ribosomes and

would have reached its maximum polysome size. However, direct testing indicates that maximal polysome size is reached only at three to five transit times, much slower than expected (203). One interprets this observation to indicate that there is a competition between ribosomes already on the mRNA with free, uncommitted ribosomes and that ribosomes which have just completed peptide chain synthesis are more efficient at reinitiation. This may reflect some tertiary steric condition or may indicate that physically the nearest ribosome is that already attached to the mRNA so that an element of an "intramolecular" reaction is favored over the "intermolecular" reaction.

However, more recent data indicate that although poly(A) tails may yield larger polysomes (and hence more efficient mRNAs), in fact the very first initiation event is also more favored on an mRNA with a poly(A) tail. By using inhibitors to block multiple initiation events, it was shown that although poly(A) tails do not enhance 48S complex formation (40 · Met-tRNA · mRNA), they do enhance the formation of 80S complex formation (198), suggesting a role in subunit joining. Confirmation of this idea was obtained by genetic studies with mutant forms of PABP and reversion analyses (252). A suppressor mutation for deletion of PABP was identified as ribosomal protein L46, a 60S subunit protein, consistent with the idea that the poly(A) with bound PABP enhances 60S subunit joining. However, perhaps even more convincing data are that this signal [poly(A) + PABP] can work in *trans*; that is, the addition of poly(A) to lysates which contain mRNAs without poly(A) tails will result in a stimulation of translation of the poly(A)$^-$ mRNA (198). However, it is not clear why poly(A)$^+$ mRNAs are not also stimulated.

A general hypothesis could be put forward that the poly(A) · PABP complex interacts with the 60S ribosomal subunit and moderately activates this subunit to enhance subunit joining. As polysomes form and peptide chain synthesis is completed, the released 60S subunit tends to associate with the poly(A) · PABP complex and thus is activated for reinitiation and favored by the intramolecular nature of this reaction. The alternative is that the poly(A) · PABP complex serves as a magnet to bring free 60S subunits into the vicinity of the mRNA, activates it, and thus increases the flow of activated 60S subunits to join with 48S preinitiation complexes. One assumes that to serve more than a single 60S subunit, the interaction between the 60S subunit and the poly(A) · PABP complex is lost at the subunit joining step.

If indeed there is a positive translational benefit to mRNAs which possess poly(A) tails, why would mRNAs exist without them? The answer would appear to apply to all the unusual characteristics of translation (reinitiation, internal initiation) or control of the level of the protein produced independently of or in conjunction with transcriptional controls. Almost all housekeeping enzymes tend to display the favored characteristics for optimal translation, and this is especially true for the overproduced proteins within a specific tissue (hemoglobin mRNA being a classic example). The expression of these mRNAs is usually not controlled individually. In contrast, proteins subject to translational control (in the presence of continued synthesis of proteins which are not) often have less than the optimal mRNA structure, which could include an inaccessible m^7G cap structure, ORFs 5′ to the authentic coding region, lack of a poly(A) tail, use of internal initiation, or use of reinitiation. These elements have been difficult to address previously because either their existence was unknown or their possible contribution to translational efficiency was small (twofold or less in vitro). Now that we are older and wiser, the improvement of experimental design and the use of genetics are likely to provide a much better understanding of the subtleties of translation and its diversity in both mechanism and regulation.

APOLOGIES, APOLOGIES, APOLOGIES

This article has been written away from home and consequently away from a storehouse of literature which could or should have been cited for this review to have been truly "authoritative." On the other hand, such extensive citations may have made it more difficult to reach the desired audience, the nonexpert in protein synthesis. For whatever omissions may have occurred, I humbly apologize. Second, it is often noted in reviews that the author's own publications are cited extensively relative to those of other workers. This reflects the ease with which authors can readily remember each tidbit of information they feel they have contributed to the field, while those tidbits from others (especially competitors) receive single citations to a recent article or a previous review. As a good rule of thumb, divide the citations to the author's work by about 5 to 10 and a reasonable balance will have been achieved. Finally, as evidenced by the section on loose ends, the mechanism and control of eukaryotic protein synthesis are under constant revision, and you have been presented with my interpretation thereof. Alternate viewpoints are clearly held, and the real enthusiast is encouraged to take in these other interpretations and more detailed descriptions on specific elements of translational control as they are presented in recent books, as well as several of the recent reviews which are more focused on regulation of translation.

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